



Elevated Temperature Does Not Substantially Modify the Interactive Effects Between Elevated CO₂ and Diel CO₂ Cycles on the Survival, Growth and Behavior of a Coral Reef Fish

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Specialty section:

This article was submitted to
Global Change and the Future Ocean,
a section of the journal
Frontiers in Marine Science

Received: 25 July 2018

Accepted: 14 November 2018

Published: 12 December 2018

Citation:

Jarrold MD and Munday PL
(2018) Elevated Temperature Does
Not Substantially Modify
the Interactive Effects Between
Elevated CO₂ and Diel CO₂ Cycles on
the Survival, Growth and Behavior
of a Coral Reef Fish.
Front. Mar. Sci. 5:458.
doi: 10.3389/fmars.2018.00458

Recent studies demonstrate that diel CO₂ cycles, such as those prevalent in many shallow water habitats, can potentially modify the effects of ocean acidification conditions on marine organisms. However, whether the interaction between elevated CO₂ and diel CO₂ cycles is further modified by elevated temperature is unknown. To test this, we reared juvenile spiny damselfish, *Acanthochromis polyacanthus*, for 11 weeks in two stable (450 and 1000 μ atm) and two diel- cycling elevated CO₂ treatments (1000 \pm 300 and 1000 \pm 500 μ atm) at both current-day (29°C) and projected future temperature (31°C). We measured the effects on survivorship, growth, behavioral lateralization, activity, boldness and escape performance (fast starts). A significant interaction between CO₂ and temperature was only detected for survivorship. Survival was lower in the two cycling CO₂ treatments at 31°C compared with 29°C but did not differ between temperatures in the two stable CO₂ treatments. In other traits we observed independent effects of elevated CO₂, and interactions between elevated CO₂ and diel CO₂ cycles, but these effects were not influenced by temperature. There was a trend toward decreased growth in fish reared under stable elevated CO₂ that was counteracted by diel CO₂ cycles, with fish reared under cycling CO₂ being significantly larger than fish reared under stable elevated CO₂. Diel CO₂ cycles also mediated the negative effect of elevated CO₂ on behavioral lateralization, as previously reported. Routine activity was reduced in the 1000 \pm 500 μ atm CO₂ treatment compared to control fish. In contrast, neither boldness nor fast-starts were affected by any of the CO₂ treatments. Elevated temperature had significant independent effects on growth, routine activity and fast start performance. Our results demonstrate that diel CO₂ cycles can significantly modify the growth and behavioral responses of fish under elevated CO₂ and that these effects are not altered by elevated temperature, at least in this species. Our findings add to a growing body of work that highlights the critical importance of incorporating natural CO₂ variability in ocean acidification experiments to more accurately assess the effects of ocean climate change on marine ecosystems.

Keywords: ocean acidification, ocean warming, lateralization, activity, fast starts, pH variability, CO₂ fluctuations

INTRODUCTION

Increasing atmospheric carbon dioxide (CO₂) levels are driving ocean warming (OW) and a decline in seawater pH, a process referred to as ocean acidification (OA) (Collins et al., 2013). Both OA and OW are expected to significantly impact the performance of marine organisms, with severe consequences for community dynamics and ecosystem functioning (Schiel et al., 2004; Doney et al., 2012; Kroeker et al., 2012; Cheung et al., 2013; Wittmann and Pörtner, 2013; Nagelkerken and Connell, 2015). However, while there has been extensive research on the effects of OA and OW in isolation, comparatively less is known about their combined effects. Understating how multiple climate change drivers affect marine organisms is vital to accurately predict future impacts (Riebesell and Gattuso, 2015).

To date, most OA experiments have tested shallow water coastal species, yet have been conducted using stable elevated CO₂ levels consistent with open ocean projections (McElhany and Busch, 2013; Wahl et al., 2016). Unlike the open ocean, CO₂ levels in shallow water habitats are not in equilibrium with the atmosphere over short time scales, with many habitats experiencing substantial fluctuations in CO₂ on a daily basis (Hofmann et al., 2011; Shaw et al., 2012; Duarte et al., 2013; Baumann et al., 2015; Challener et al., 2016). These diel CO₂ cycles are driven primarily by the processes of photosynthesis and respiration across a day–night cycle, but are also influenced by a variety of local hydrodynamic factors (Falter et al., 2013; Waldbusser and Salisbury, 2014). Importantly, a number of studies have demonstrated that diel CO₂ cycles can significantly modify organismal responses to OA, highlighting the critical importance of incorporating natural CO₂ variability in OA experiments (e.g., Cornwall et al., 2013; Frieder et al., 2014; Ou et al., 2015; Jarrold et al., 2017; Enochs et al., 2018; Wahl et al., 2018). However, it is currently unknown if the interaction between elevated CO₂ and diel CO₂ cycles will be modified by elevated temperature.

Marine fish were initially expected to be resilient to OA as they can tightly defend their internal pH under elevated CO₂ conditions *via* active control of acid-base relevant ions (Wood et al., 1990; Baker et al., 2009; Heuer and Grosell, 2014). However, the increased energetic costs associated with this compensatory acid-base regulation could have detrimental effects on growth and survival, particularly in early life stages where the cost of homeostasis is greater (Brauner, 2009). While some studies have indeed reported negative effects of OA on growth and survival in the early life stages of marine fish (e.g., Miller et al., 2012; Murray et al., 2014; Stiasny et al., 2016; Gobler et al., 2018), others have observed neutral (e.g., Munday et al., 2011; Bignami et al., 2013; Frommel et al., 2013; Watson et al., 2018) and even positive effects (e.g., Munday et al., 2009b; Cattano et al., 2017). To date, only two studies have investigated how diel CO₂ cycles will interact with elevated CO₂ levels to affect the survival and growth of marine fishes under OA conditions. Diel CO₂ cycles (450–2000 μ atm) were shown to alleviate the negative effects of elevated CO₂ (1000 μ atm) on growth of larval pink salmon,

Oncorhynchus gorbuscha (Ou et al., 2015). In contrast, diel CO₂ cycles (1000 \pm 300 and 500 μ atm) had no significant effect on growth and survival in two species of coral reef fish (Jarrold and Munday, 2018).

Some of the most notable effects of elevated CO₂ on marine fish have been the impacts on ecologically important behaviors (Nagelkerken and Munday, 2016; Cattano et al., 2018). A wide array of behavioral responses, across a range of species, have now been shown to be altered under elevated CO₂, including: predator avoidance/prey detection behavior (e.g., Pistevo et al., 2015; Sundin and Jutfelt, 2015; Munday et al., 2016; McMahon et al., 2018) escape responses (e.g., Allan et al., 2013; Munday et al., 2016), activity/boldness levels (e.g., Jutfelt et al., 2013; Hamilton et al., 2014; Munday et al., 2014) and lateralization (e.g., Jutfelt et al., 2013; Welch et al., 2014; Lopes et al., 2016; Schmidt et al., 2017). Nevertheless, species/trait-specific responses are also evident, with other studies reporting no impacts of elevated CO₂ on fish behavior (e.g., Jutfelt and Hedgärde, 2013; Heinrich et al., 2016; Cattano et al., 2017; Schmidt et al., 2017; Sundin et al., 2017; Laubenstein et al., 2018). The changes in behavioral responses under elevated CO₂ observed in laboratory experiments are expected to have significant ecological consequences for fish populations through effects on recruitment, dispersal, predator-prey/competitive interactions and habitat preference (Nagelkerken and Munday, 2016), although such effects may also be offset by compensatory and indirect effects in more diverse communities (Munday et al., 2014; Goldenberg et al., 2018). Only two studies have investigated how diel CO₂ cycles will interact with elevated CO₂ levels to affect the behavioral performance of marine fishes under OA conditions. Behavioral impairments in two species of coral reef fish under elevated CO₂ were less severe, or absent, in the presence of diel-cycling CO₂ regime (1000 \pm 300 and 500 μ atm) (Jarrold et al., 2017). In contrast, diel CO₂ cycles (587–1066 μ atm) had no significant effect on behavioral responses of juvenile blacksmith, *Chromis punctipinnis* (Kwan et al., 2017).

In contrast to elevated CO₂, the effects of elevated temperature on the growth and survival of marine fish are more consistent and predictable. Increases in temperature can result in increased growth if the species is living below its thermal optimum (Green and Fisher, 2004; McLeod et al., 2015; Moyano et al., 2016; Gobler et al., 2018). By contrast, growth and survivorship are often reduced at temperatures above the thermal optimum, as metabolic demands exceed capacity (Pörtner and Knust, 2007; Munday et al., 2008; Todd et al., 2008; Neuheimer et al., 2011). Elevated temperatures have also been shown to impact a range of behavioral responses, in some cases having stronger effects than elevated CO₂, particularly behaviors which are closely linked to physiological condition such as routine activity and escape performance (Biro et al., 2010; Allan et al., 2017; Schmidt et al., 2017; Laubenstein et al., 2018; Watson et al., 2018).

Considerably less is known about how OA and OW will interact to affect the performance of marine fish. However, a few studies have demonstrated that responses to one stressor can be modified by the presence of another. For example,

the positive effects of elevated temperature on growth of larval flat fish, *Solea senegalensis*, were reduced when CO₂ was also elevated (Pimentel et al., 2014). Furthermore, while elevated temperature was shown to reduce growth and survival in larval Atlantic herring, *Clupea harengus*, the effects of elevated CO₂ were shown to be temperature dependent (Sswat et al., 2018; see also Gobler et al., 2018). From a behavioral perspective, elevated CO₂ and temperature interacted synergistically on predation rate, but antagonistically on predator selectivity of the dottyback, *Pseudochromis fuscus* (Ferrari et al., 2015). Furthermore, elevated temperature reduced the effects of elevated CO₂ on relative laterization in the damselfish *Pomacentrus wardi* (Domenici et al., 2014). Collectively, these studies highlight the importance of studying the combined effects of OA and OW if we are to accurately predict the impacts of climate change on marine fish and the associated ecological consequences. It is currently unknown how elevated temperature will modify the interaction between elevated CO₂ and diel CO₂ cycles to affect the performance of marine fishes.

Here, we investigated the effects of elevated CO₂, diel CO₂ cycles and elevated temperature on the survival, growth and behavior of a coral reef fish, the spiny damselfish, *Acanthochromis polyacanthus*. Diel CO₂ cycles in some shallow coral reef habitats have been shown to range up to ± 600 μatm around the mean (Shaw et al., 2012), although smaller ranges ($< \pm 200$ μatm) are more common (Kayanne et al., 1995; Manzello, 2010; Albright et al., 2013; Kline et al., 2015). Importantly, diel CO₂ cycles on coral reefs are predicted to be amplified up to threefold over the next century (Shaw et al., 2013), and thus cycles with greater magnitude may become more common by the year 2100. To test if elevated temperature alters the effects of elevated CO₂ and diel CO₂ cycles on reef fishes, we reared juvenile damselfish for 11 weeks at control (450 μatm), stable elevated (1000 μatm) and two diel-cycling elevated (1000 \pm 300 μatm and 1000 \pm 500 μatm) CO₂ treatments at both 29°C (current-day average summer temperature) and 31°C (year 2100 prediction; Collins et al., 2013) (Table 1 and Supplementary Figure 1). We compared survivorship and growth among treatments. We also tested behavioral lateralization, routine activity, boldness and escape performance (i.e., fast starts) of fish from each treatment,

as past research has shown that these traits can be affected by both elevated CO₂ and temperature.

MATERIALS AND METHODS

Study Species and Brood-Stock Maintenance

Acanthochromis polyacanthus is common on coral reefs in the Indo-west Pacific. They are demersal spawners, laying clutches of eggs within small caves and crevices in the reef matrix. Eggs hatch into small juveniles, with both parents providing care to the eggs and offspring for up to 45 days post-hatching (Kavanagh, 2000). *A. polyacanthus* can be bred and reared in captivity with high success, which has led to their establishment as a model for investigating the potential impacts of OA and OW on coral reef fishes (Munday et al., 2008, 2011; Donelson et al., 2012; Welch et al., 2014; Heuer et al., 2016; Jarrold et al., 2017).

Adult *A. polyacanthus* were collected using hand nets from the Bramble Reef area (site 1: 18°22'S, 146°40'E; site 2: 18°25'S, 146°40'E) of the Great Barrier Reef in July 2015. Fish were transported to an environmentally controlled aquarium research facility at James Cook University (JCU) (Townsville, QLD, Australia) where they were housed as breeding pairs in 60 L aquaria at temperature conditions matching the collection location. Breeding pairs were maintained under stable, ambient CO₂ (~ 490 μatm). Temperatures were increased at a rate of 0.5°C per week until the summer breeding temperature of 29°C was reached in the first week of December 2016. Breeding pairs were provided with half a terracotta pot to act as a shelter and spawning site. Aquaria were checked each morning for the presence of newly laid clutches of eggs. Pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture Nutrition NRD 12/20) once daily outside the breeding season and twice daily during the breeding season.

Experimental Design

The experimental part of the study was carried out at the National Sea Simulator (SeaSim) facility at the Australian Institute of Marine Science (AIMS) (Cape Cleveland, QLD, Australia). Clutches of juveniles were transferred from JCU to the

TABLE 1 | Experimental seawater parameters.

CO ₂ treatment	Temperature treatment	Mean pCO ₂ (μatm)	Min pCO ₂ (μatm)	Max pCO ₂ (μatm)	Temperature (°C)	Salinity	TA ($\mu\text{mol kg}^{-1}$)
450	29	435 \pm 38	425 \pm 36	477 \pm 163	28.8 \pm 0.2	35.4 \pm 0.4	2325 \pm 33
450	31	475 \pm 44	459 \pm 31	527 \pm 224	31.1 \pm 0.3	35.4 \pm 0.4	2306 \pm 51
1000	29	992 \pm 19	956 \pm 74	1035 \pm 95	28.8 \pm 0.2	35.4 \pm 0.4	2325 \pm 32
1000	31	981 \pm 8	958 \pm 42	1002 \pm 23	31.1 \pm 0.3	35.4 \pm 0.4	2310 \pm 48
1000 \pm 300	29	1009 \pm 25	653 \pm 25	1329 \pm 89	28.8 \pm 0.2	35.4 \pm 0.4	2318 \pm 38
1000 \pm 300	31	1034 \pm 33	668 \pm 27	1349 \pm 97	31.1 \pm 0.3	35.4 \pm 0.4	2318 \pm 36
1000 \pm 500	29	1060 \pm 34	521 \pm 43	1544 \pm 80	28.8 \pm 0.2	35.4 \pm 0.4	2323 \pm 32
1000 \pm 500	31	1079 \pm 49	556 \pm 64	1570 \pm 118	31.1 \pm 0.3	35.4 \pm 0.4	2314 \pm 39

Values are means \pm 1 SD for mean, min and max pCO₂, temperature, salinity and total alkalinity (TA), pCO₂ data is based on one reading every 10 min. Temperature data is based on one reading per hour. Salinity and TA data are based on weekly measurements.

experimental system at AIMS at 1 day post hatch (dph) where they were reared for 11 weeks at two stable (450 and 1000 μatm) and two cycling (1000 \pm 300 and 1000 \pm 500 μatm) CO₂ treatments at both 29 and 31°C. A total of four offspring clutches were used in the experiment, sourced from two breeding pairs (two clutches per pair). Each clutch was split between the CO₂ and temperature treatments in duplicate tanks (15–25 fish per tank with tanks on different systems). Juvenile *A. polyacanthus* were fed 6mL of freshly hatched *Artemia naupli* (\sim 4000 mL⁻¹) for the first 4 days post hatch (dph). From 5 to 28 dph they were fed daily (0.12 g) on a weaning fish feed (INVE Aquaculture Nutrition Wean-S 200–400 μm) and from 29 to 42 dph they were fed daily (0.15 g) on a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8). Feeding was increased to 0.2 g after 42 dph and to 0.35 g after 70 dph.

Fast start trials were performed 28–35 dph, lateralization trials were performed 42–49 dph and activity/boldness trials were performed 63–70 dph. Fast start and lateralization trials were only performed on three clutches due to logistic constraints. Behavioral trials took place across four consecutive days with the temperature treatment tested alternating between days. This allowed the room temperature to be adjusted accordingly, thus ensuring a stable testing temperature was maintained. Fish were randomly selected for each behavioral trial, which means that some fish were likely tested for more than one behavioral trial, and the time between trials was to ensure that they recovered from any stress between trials. To ensure a fish was not tested twice within a trial, fish were placed in an isolation chamber in their experimental tank after testing for the rest of the day. All behavioral trials were performed between 09:00 and 17:00. Fish were gently transferred to the behavioral arenas using a glass beaker to minimize handling stress. Fish from each CO₂ treatment were tested at random times throughout the day to account for any possible time of day effects in the fluctuating treatments. At the end of the rearing period all fish were euthanised with clove oil anesthetic. Each fish was blotted dry, weighed (nearest mg) on an analytical balance (AX224, Sartorius, Bradford, PA, United States) and photographed in a lateral position next to a ruler. Standard length (SL) to the nearest 0.1 mm was estimated for each fish from the digital photographs using ImageJ software¹. Research was carried out under approval of the James Cook University animal ethics committee (permit: A2210) and according to the University's animal ethics guidelines.

Experimental System

The experimental setup used at SeaSim comprised of 16 independent flow-through systems (4 systems *per* CO₂ treatment). Each system supplied ultra-filtered seawater (0.04 μm), at either 29 or 31°C, to three custom-made 50 L experimental tanks at the rate of 50 L h⁻¹. Thus, there was total of 48 tanks (6 tanks *per* CO₂/temp treatment). The experimental tanks were placed in individual temperature-controlled water baths to ensure temperature stability (\pm 0.1°C). Treatments and tank replicates were randomly positioned in the experimental

room. **Supplementary Figure 2** shows a schematic diagram of an individual system.

*p*CO₂ and temperature levels were controlled through a custom designed Model Predictive Control running on a micro-programmable logic controller (PLC) (Series S7-1500, Siemens, Australia). The micro-PLC was integrated with the general SeaSim control system, to provided SCADA (Siemens WinCC) accessibility and data archiving. The CO₂ feedback for each of the replication lines was provided via non-dispersive infrared measurements. On each system, water from one tank was delivered to an equilibrator (Seasim, AIMS design, custom built) *via* an in-tank submersible pump (Universal Pump 1260, EHEIM, Deizisau, Germany) where the air space in the chamber maintains an equilibrium with the CO₂ of the experimental water. The air was constantly delivered to a non-dispersive infrared CO₂ analyzer (Telaire T6613, Amphenol, Australia) that provided live feedback to the PLC. The CO₂ analyzers were calibrated monthly using certified calibration gas mixtures at 0, 600, and 2000 ppm. The control system delivered pure CO₂ through Gas Mass Flow Controllers (GFC17 series, Aalborg, Orangeburg, NY, United States) according to the profiling schedule designed for the *p*CO₂ treatment and the feedback signal coming from the experimental tanks. CO₂ was dissolved in the flow-through water by membrane contactors (Membrana Liqui-Cel 2.5 \times 8 Extra-Flow, 3M, United States). Throughout the experiment incoming coastal water had a *p*CO₂ ranging between 500 and 550 μatm . Thus, to achieve a control *p*CO₂ level closer to 450 μatm , membrane contactors (Membrana Liqui-Cel 4 \times 28 Extra-Flow) were used to remove CO₂, using CO₂ – depleted air as sweep gas. Total alkalinity was measured from a random tank on half of the systems each week (the systems measured alternated each week) using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). Mean values for seawater parameters are presented in **Table 1**. **Supplementary Figure 1** illustrates the shape of the CO₂ profiles used.

Behavioral Assays

Fast Starts

Fast start trials were performed using methods similar to those described by Munday et al. (2016). The setup consisted of a circular experimental arena (diameter 210 mm) placed inside an opaque rectangular container (550 \times 410 \times 320 mm). A polystyrene lid was placed on top of the container with a white PVC tube (40 mm \times 300 mm) inserted through the middle. An electromagnet was attached to the top of the PVC tube to which a weighted stimulus was attached via a metal disk. The arena was illuminated by an LED strip placed around the outside of the container. The container was filled with water from the treatment fish were reared in to a depth of 50 mm to reduce movement in the vertical plane. Once introduced into the arena fish were given a 3 min habituation period. After this period, a fast start was elicited by turning off the electromagnet, thus releasing the stimulus. A length of fishing line attached to the weight caused it to stop when the tip of the weight only just touched the surface of the water after release from the magnet. To provide a sudden

¹<http://rsb.info.nih.gov/ij/>

stimulation and allow calculation of the response latency the bottom edge of the PVC tube was placed just above the water level. This way there was no visual stimulation before mechanical stimulation. Fish were only startled when they were more than two body lengths from the arena's edge to minimize edge effects on escape responses. Responses to the stimulus were filmed as a silhouette from below with a high-speed camera (Casio EX-ZR2000; 480 fps). To do this, the experimental setup was placed on a wooden frame with a mirror placed inside at 45° degrees. The front of the frame was covered with black plastic sheet, to minimize external disturbance, with a small hole cut out in the middle for the camera lens. Five to seven fish per tank were tested. Video recordings were analyzed with the observer blind to treatment using IMAGEJ software and calibrated against the base of the PVC tube. The very front of the fish was used as the reference point for tracking. From the videos, we quantified non-locomotor traits (response latency and directionality) and locomotor traits (turning rate, mean escape speed, maximum escape speed and escape distance). The moment the stimulus weight hit the water was benchmarked by the first detectable water disturbance in the video. Fish escape variables were only measured if a C-start was initiated.

Non-locomotor variables

- (1) Response latency (in ms) was measured as the time interval between the stimulus onset and the first detectable movement leading to the escape of the animal.
- (2) Directionality: escape responses were divided into 'away' and 'toward' responses when the first detectable movement of the head was oriented away and toward the stimulus, respectively.

Locomotor variables

- (1) Turning rate (degrees ms⁻¹) was measured as the angle turned before the fish swam away after the onset of the response.
- (2) Mean response speed (ms⁻¹) was measured as the distance covered within a fixed time (24 ms) which corresponds to the average duration of the first two tail flips of the tail (the first two axial bends, i.e., stages 1 and 2 defined based on Domenici and Blake (1997), which is the period considered crucial for avoiding predator ambush attacks).
- (3) Maximum speed (ms⁻¹) was measured as the maximum speed reached at any time during the response.
- (4) Escape distance was measured as the straight-line distance between the position of the fish at the onset of a C-start and the position of the fish after 24 ms.

Behavioral Lateralization

Lateralization in juvenile *A. polyacanthus* was determined using a detour test in a two-way T-maze using methods described by Jarrold et al. (2017). The two-way T-maze consisted of an experimental arena (60 cm × 30 cm × 20 cm), with a runway in the middle (25 cm × 2 cm, length × width), and at both ends of the runway (2 cm ahead of the runway) an opaque barrier (12 cm × 12 cm × 1 cm) was positioned perpendicular to the runway. The maze was filled to a depth of 4 cm with the respective

treatment water of the fish being tested, being changed after each trial. A single fish was placed at one end of the T-maze and given a 3 min habituation period, during which time it could explore the apparatus. At the end of the habituation period the fish was gently guided into the runway using a plastic rod with the observer standing directly behind the fish (the plastic rod was never placed closer than approximately twice the body length of the fish). At this point to minimize human interference affecting direction turned the observer slowly stepped back from the maze and the fish was allowed to swim to the end of the runway. In instances when a fish did not swim to the end, encouragement was provided by gently moving the plastic rod around at the beginning of the runway. Consecutive runs were carried out until 10 successful runs per fish had been recorded. Six to eight fish were tested per tank. To account for any possible asymmetry in the maze, turns were recorded alternately on the two ends of the runway. Turning preference (i.e., bias in left or right turns) at the population level was assessed using the relative lateralization index (L_R , from -100 to +100, indicating complete preference for left and right turning, respectively) according to the following formula: $L_R = [(\text{Turn to the right} - \text{Turn to the left}) / (\text{Turn to the right} + \text{Turn to the left})] * 100$. The strength of lateralization (irrespective of its direction) was also assessed at the individual-level using the absolute lateralization index L_A [ranging from 0 (an individual that turned in equal proportion to the right and to the left) to 100 (an individual that turned right or left on all 10 trials)]. Trials were filmed, and fish were scored from video analysis with the observer blind to treatment. Direction choice was recorded as the first direction turned when the fish exited the runway.

Routine Activity and Boldness

Routine activity and boldness were determined using an open field test with methods similar to those described by Laubenstein et al. (2018). The setup consisted of a round, white plastic arena (26 cm diameter, 6 cm height) placed inside a white plastic bin (52 cm length, 32 cm width, 34 cm height), which was opaque to minimize visual disturbance for the fish but allowed light through for filming. Two wooden planks (L = 60 cm, H = 9 cm, W = 2 cm) were placed across the top of each plastic bin with a sheet of white corflute on top which had a small circular hole cut into its center, where a video camera (Casio EX-ZR2000) was placed. This was done so that the whole arena fitted into the camera's field of view. Fish were tested in their treatment water at a depth of 5 cm. To begin a trial, a fish was transferred into the center of the arena by gently transferring it with a beaker to minimize stress. The camera was turned on, and the fish was filmed for 15 min. Six to nine fish per tank were tested. Activity and space use in the tank were determined from the video using motion-tracking software (Lolitrack v4.1.0, Loligo Systems, Tjele, Denmark). Before each video analysis, a circular arena was drawn within the test arena, with the same central point, but which was 18 cm in diameter, or approximately 1.5 standard body lengths away from the edges of the test arena. This "center zone" was used to quantify boldness, based on the idea that an open field is considered dangerous, and that venturing into the inner zone represents boldness, or the willingness to undertake risk. Therefore, we quantified boldness

by measuring the number of visits made to the center zone and time spent in the center zone. The routine activity parameters quantified by the software were: time active (%) and average swimming velocity (cm s⁻¹). All videos were analyzed blind to treatment. The first 5 min of each video were discarded to allow some habituation time.

Data Accessibility

The datasets generated during the current study are available from the corresponding author on request or via the Tropical Research Data Hub (doi: 10.25903/5bd7c7f552897).

Statistical Analysis

All analyses were conducted using R version 3.4.0. (R Core Team, 2017) using the following packages: nlme, lme4 and glmmTBM. In all cases, except for survival data, mixed effects models were used so that random effects could be incorporated. In all mixed effects models, pCO₂ and temperature treatment were fixed factors, with clutch and tank as random factors with tank nested within clutch. Initially, fully interactive models were run. However, non-significant interaction terms were gradually removed. Final model choice was confirmed based on Akaike information criterion (AIC). When a CO₂ effect was observed pair-wise comparisons were conducted using Tukey's tests using the 'multcomp' package. When a significant CO₂ * temperature interaction was found (survival data only) planned contrasts were performed. For planned comparison we compared pCO₂ treatments across each temperature treatment separately and temperature treatments within a pCO₂ treatment.

Survival and Growth

The effects of CO₂ and temperature on juvenile survival were tested using a generalized linear model (GLM) with a binomial distribution (due to the data being proportional), weighted to the starting number of fish in each tank. The effects of CO₂ and temperature on wet weight and standard length were analyzed using linear mixed effects models (LMEs). The number of fish remaining in each tank at the end of the experiment was included as a covariate. Additive models were used (number + CO₂ + temperature).

Fast Starts

The effects of CO₂ and temperature on latency to respond to the stimulus were tested using an LME with distance of the fish from the center of the experimental arena when the stimulus hit the water as a covariate. The effects of CO₂ and temperature on direction turned were analyzed with a generalized linear mixed model (GLMM) with a binomial distribution. The effects of CO₂ and temperature on turning rate, mean escape speed, maximum escape speed and escape distance were tested using LMEs with total length included as a covariate for mean and maximum escape speed and escape distance. Additive models were used for latency, direction turned, turning rate and escape distance. For mean and maximum escape speed the model tested the interactive effects between total length and temperature and the additive effect of CO₂ (body length * temperature + CO₂).

Behavioral Lateralization

GLMMs with binomial distributions and weighted to the number of runs (10) were used to test the effects of CO₂ and temperature on absolute lateralization (L_A) and relative lateralization (L_R) due to the proportional nature of the data. Additive models were used (CO₂ + temp).

Routine Activity and Boldness

A GLMM with a binomial distribution and weighted to the length of time of the trial (10 min) was used to test the effects of CO₂ and temperature on percentage time active. The effects of CO₂ and temperature on velocity and time spent in the center zone (fish that did not visit the center zone were excluded from analysis) were tested using LMEs. Finally, a mixed-effects hurdles model was used to test the effects of CO₂ and temperature on number of visits to the center zone due to the high number of zeros present in the dataset. Total length was included as a covariate for all traits. Additive models were used for time active, velocity and number of visits to the center zone. An interactive model was used for time spent in center zone (body length * temperature * CO₂).

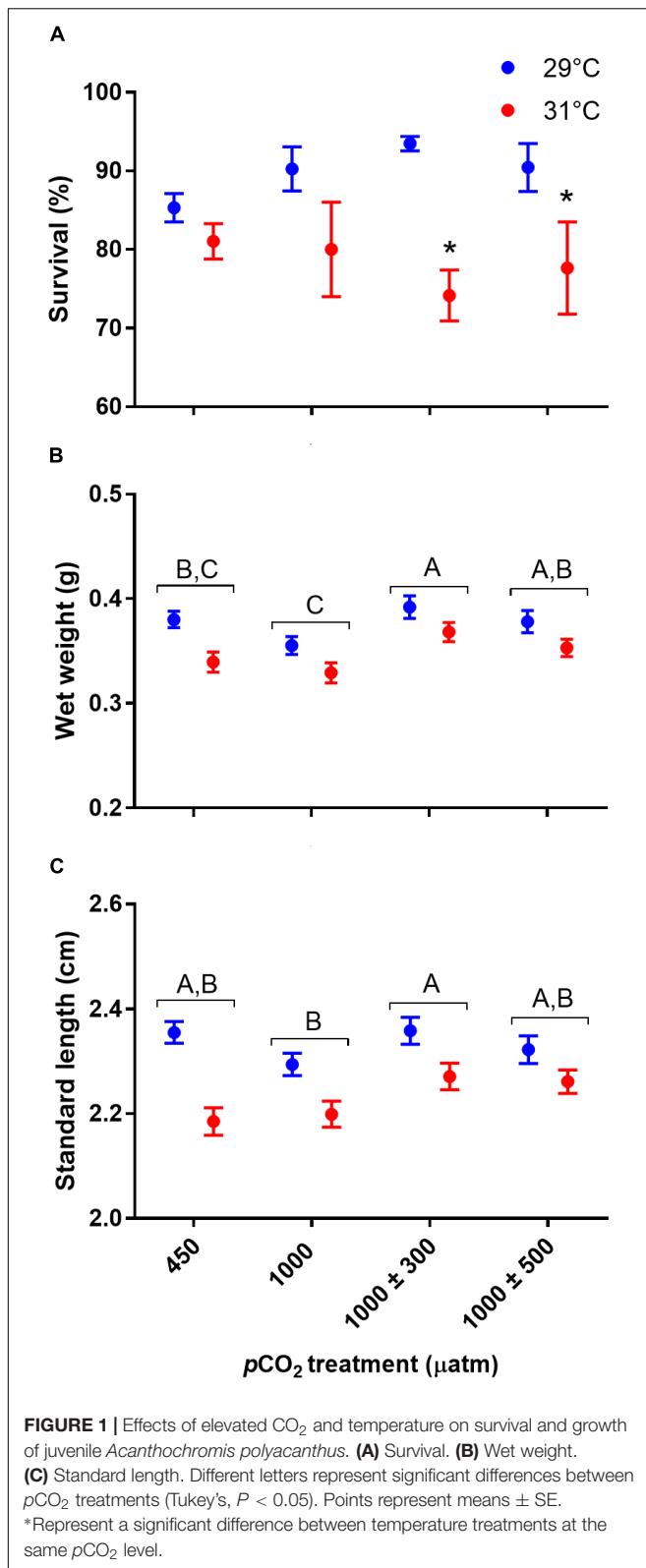
RESULTS

Survival and Growth

Elevated temperature (Figure 1A, $\chi^2 = 29.59$, df = 1, $P < 0.001$) but not elevated CO₂ ($\chi^2 = 0.74$, df = 3, $P = 0.863$) had an overall negative effect on survival of juvenile *A. polyacanthus*. Survivorship from hatching to 11 weeks post hatch decreased from 89.9% at 29°C to 78.2% at 31°C. In addition, a significant interactive effect between CO₂ and temperature was observed ($\chi^2 = 7.85$, df = 3, $P = 0.049$). Specifically, elevated temperature had a negative effect on survival in the two diel-cycling CO₂ treatments (max. $P = 0.047$), but not under control or stable elevated CO₂ conditions (max. $P = 0.194$).

Wet weight of juvenile *A. polyacanthus* was significantly affected by CO₂ (Figure 1B, $F_{3,54} = 6.32$, $P < 0.001$) and temperature (Figure 1B, $F_{1,54} = 58.90$, $P < 0.0001$) treatment. There was a trend of decreased (−4.7%) wet weight of fish reared under stable elevated CO₂ compared to those reared under control conditions, although this was non-significant ($P = 0.506$). However, fish reared under diel-cycling elevated CO₂ conditions were significantly heavier (+11.2% for ± 300 μatm and +6.6% ± 500 μatm CO₂ respectively) compared to those reared under stable elevated CO₂ conditions (min. $P = 0.035$). Fish reared under the smaller CO₂ fluctuations were also significantly heavier (+5.9%) compared to fish reared under control conditions ($P = 0.033$). Elevated temperature had a negative effect on wet weight of juvenile *A. polyacanthus*, decreasing it by 7.8% under 31°C. Finally, the number of fish remaining in the tank at the end of the experiment had a significant effect on wet weight, with heavier fish in tanks with fewer fish ($F_{1,54} = 42.10$, $P < 0.0001$).

Standard length of juvenile *A. polyacanthus* was significantly affected by CO₂ (Figure 1C, $F_{3,54} = 3.46$, $P = 0.023$) and temperature (Figure 1C, $F_{1,54} = 69.33$, $P < 0.0001$) treatment.



Fish reared at 1000 \pm 300 μ atm CO₂ were significantly longer (+3.2%) compared to those reared under stable elevated CO₂ ($P = 0.022$). However, neither were significantly different

compared to control fish (min. $P = 0.144$). Finally, fish reared under 1000 \pm 500 μ atm CO₂ were of comparable length to fish from all other CO₂ treatments (min. $P = 0.133$). Elevated temperature had a significant negative effect on length of juvenile *A. polyacanthus*, decreasing it by 4.5% under 31°C. The number of fish remaining in the tank at the end of the experiment had a significant effect on standard length, with fish reaching a longer size in tanks with fewer fish ($F_{1,54} = 18.08$, $P = 0.0001$).

Behavioral Lateralization

Absolute lateralization (L_A) was significantly influenced by CO₂ treatment (Figure 2, $\chi^2 = 17.57$, $df = 3$, $P < 0.001$) but not temperature ($\chi^2 = 0.37$, $df = 1$, $P = 0.541$). Juveniles reared under stable elevated CO₂ were less lateralized compared to those reared at control levels ($P < 0.001$). L_A of juveniles reared at 1000 \pm 300 μ atm CO₂ was partially restored being intermediate to fish reared under control and stable elevated CO₂ (min. $P = 0.060$). L_A of juveniles reared at 1000 \pm 500 μ atm CO₂ was fully restored to control levels being significantly greater than those reared at stable elevated CO₂ ($P = 0.021$). Relative lateralization (L_R) was unaffected by CO₂ ($\chi^2 = 1.67$, $df = 3$, $P = 0.644$) or temperature ($\chi^2 = 0.15$, $df = 1$, $P = 0.697$).

Routine Activity and Boldness

Percentage time active was significantly influenced by CO₂ (Figure 3A, $\chi^2 = 11.99$, $df = 3$, $P = 0.007$) and temperature (Figure 3A, $\chi^2 = 25.51$, $df = 1$, $P < 0.001$) treatments. Fish reared at 1000 \pm 500 μ atm CO₂ were significantly less active (−10.2%) than fish reared under control conditions ($P = 0.005$). No other significant differences between pCO₂ treatments were observed (min. $P = 0.059$). Elevated temperature had a negative effect on percentage time active, decreasing from 74.4% at 29°C to 64.9% at 31°C. Average velocity was also significantly affected by CO₂ (Figure 3B, $F_{3,441} = 5.15$, $P = 0.002$) and temperature (Figure 3B, $F_{1,441} = 16.66$, $P < 0.001$) treatment. Fish reared at

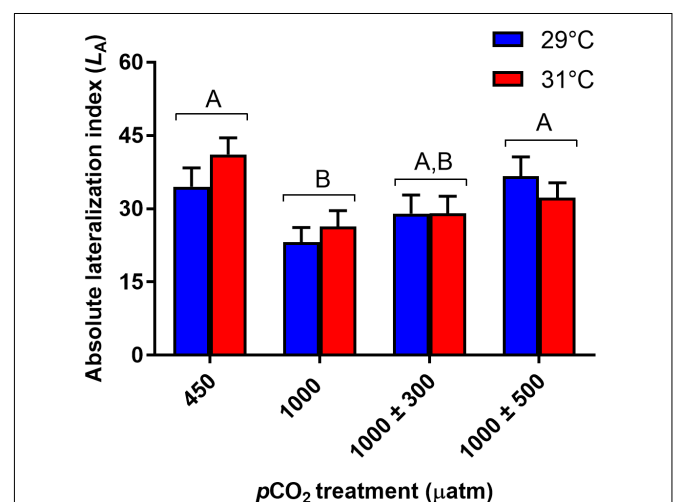
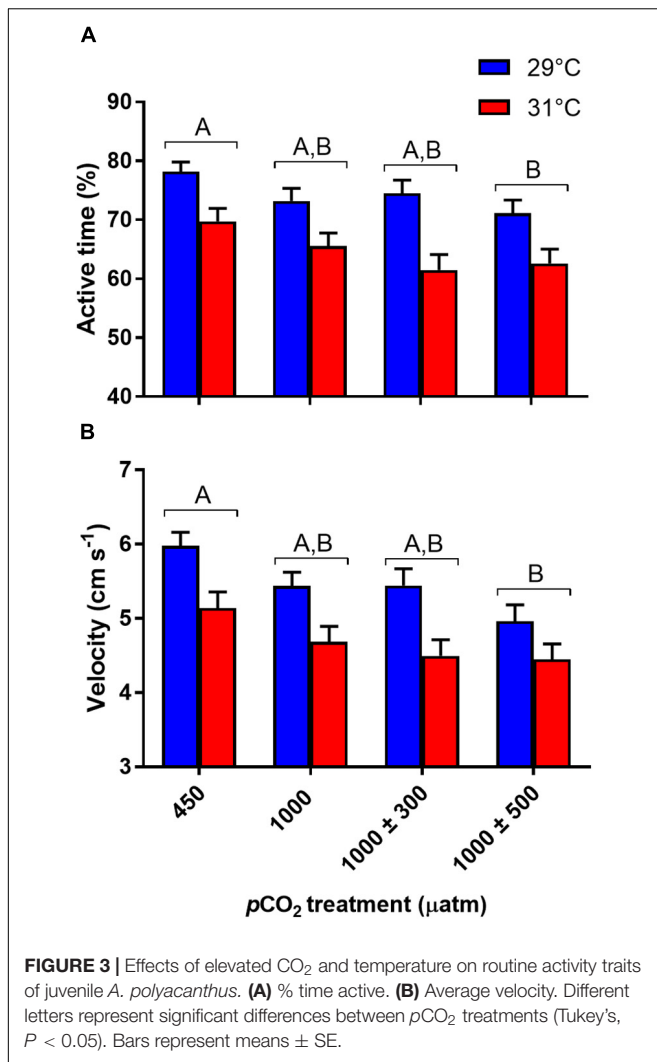


FIGURE 2 | Effects of elevated CO₂ on absolute lateralization (L_A) in juvenile *A. polyacanthus*. Different letters represent significant differences between pCO₂ treatments (Tukey, $P < 0.05$). Bars represent means \pm SE.

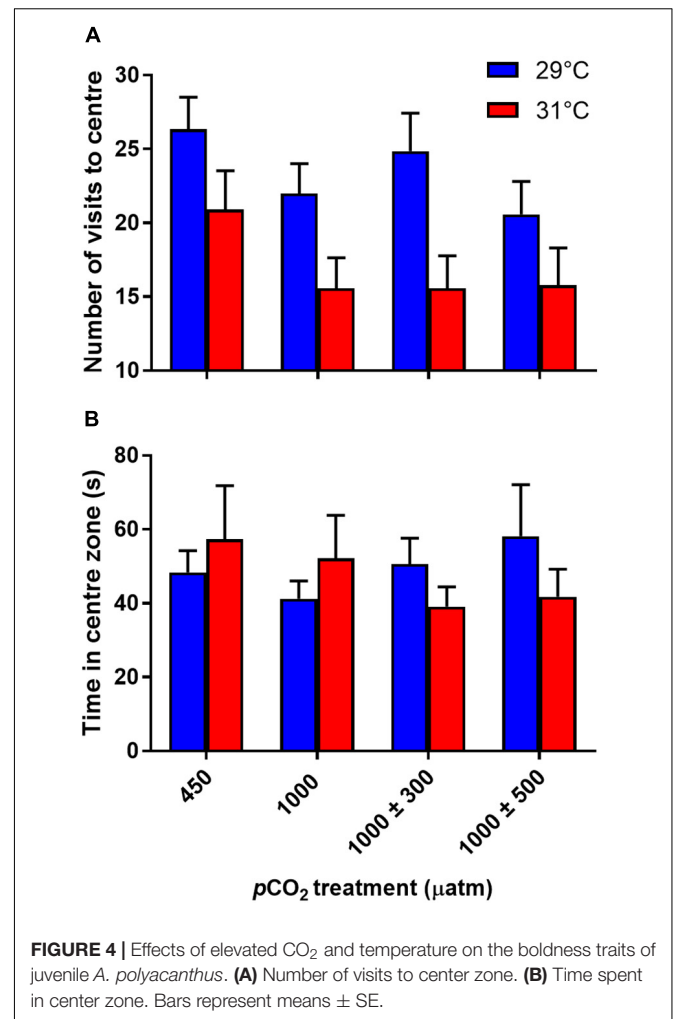


1000 \pm 500 μ atm CO₂ swam at lower speeds (-15.9%) compared to control fish ($P = 0.001$). Average velocity was negatively impacted by elevated temperature, decreasing by 14.4% at 31°C compared to fish reared at 29°C . Finally, total body length had a significant effect on average velocity ($F_{1,441} = 12.46$, $P < 0.001$), with longer fish swimming faster.

Number of visits to the center zone was not significantly influenced by CO₂ treatment (**Figure 4A**, min. $P = 0.130$), but was affected by temperature (**Figure 4A**, $P = 0.010$). Fish reared at 31°C made fewer visits to the center zone. There was no effect of CO₂ (**Figure 4B**, $F_{3,58} = 0.07$, $P = 0.974$) or temperature (**Figure 4B**, $F_{1,336} = 0.001$, $P = 0.974$) treatment on time spent in the center zone.

Fast Starts

Latency in response to the stimulus was unaffected by CO₂ (**Figure 5A**, $F_{3,42} = 1.48$, $P = 0.234$) or temperature (**Figure 5A**, $F_{1,238} = 0.58$, $P = 0.448$) treatment. The distance fish were from the center of the arena had a significant effect on escape latency with fish closer the center responding more quickly to the

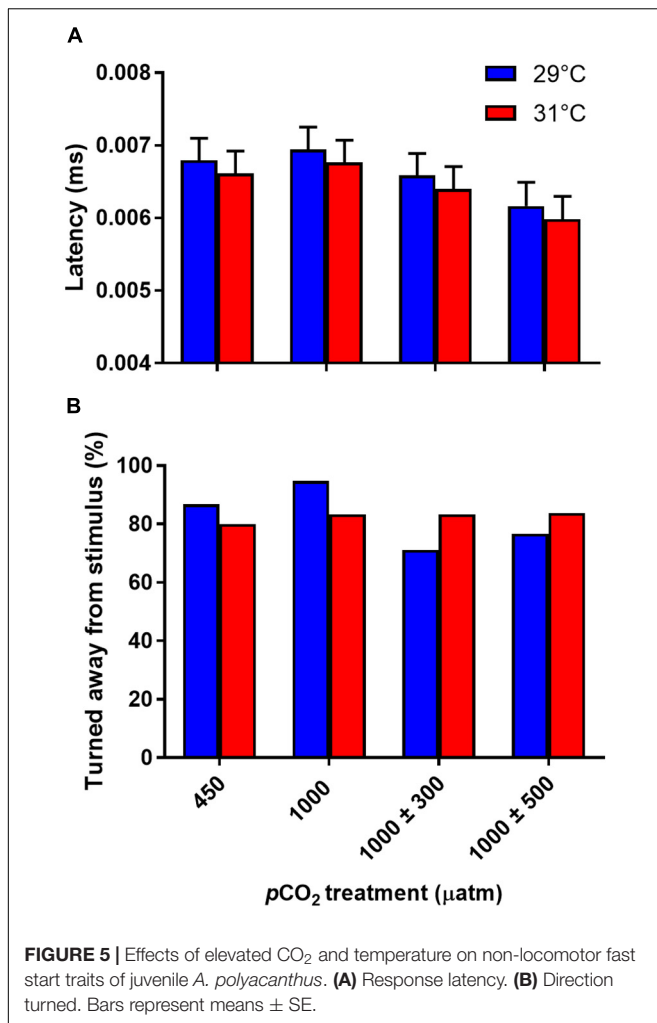


stimulus ($F_{1,238} = 9.68$, $P = 0.002$). Direction of response was also unaffected by CO₂ (**Figure 5B**, $\chi^2 = 4.08$, $df = 3$, $P = 0.252$) or temperature (**Figure 5B**, $\chi^2 = 0.00$, $df = 1$, $P = 0.988$) treatment.

All locomotor traits (turning rate, mean escape speed, maximum escape speed and escape distance) were unaffected by CO₂ treatment (max. $F_{3,42} = 1.54$, $P = 0.217$) but significantly affected by temperature (**Figures 6A–D**, min. $F_{1,237} = 4.09$, $P = 0.044$). Fish reared at 31°C turned at a faster rate ($+8.1\%$), had a greater mean ($+4.2\%$) and maximum ($+5.2\%$) escape speed and a greater escape distance ($+6.9\%$) compared to fish reared at 29°C . Total body length had a significant positive effect on mean escape speed, maximum escape speed and escape distance (min. $F_{1,237} = 11.51$, $P < 0.001$).

DISCUSSION

Recent studies have shown that diel CO₂ cycles can significantly modify the effects of ocean acidification conditions on marine organisms. However, whether the interaction between elevated CO₂ and diel CO₂ cycles is further modified by elevated temperature is unknown. Here, we report several independent



effects of elevated CO₂, and interactions between elevated CO₂ and diel CO₂ cycles, that were mostly unaffected by elevated temperature. Our results show that diel CO₂ cycles alleviated the negative effect elevated CO₂ had on growth and absolute lateralization but had a negative impact on routine activity. A significant interaction between CO₂ and temperature was only observed for survivorship, with lower survival in the two diel-cycling elevated CO₂ treatments observed at elevated temperature (31°C). Finally, elevated temperature had significant independent effects of growth, routine activity and fast start performance.

Survival and Growth

CO₂ Effects

Survival and growth of juvenile *A. polyacanthus* were not significantly affected by stable elevated CO₂ conditions, although there was a trend of decreased growth compared with control fish. Previous experiments testing the effects of stable elevated CO₂ on fish early life stages have produced highly variable and species-specific results, with negligible, negative and positive effects on survival and growth all reported

(Heuer and Grosell, 2014). The sensitivity of marine fish to OA conditions has often been linked to the environmental CO₂ levels they experience in natural habitats, with tolerant species generally occurring in areas that have daily and seasonal CO₂ cycles, and thus may already be physiologically adapted to elevated CO₂ (e.g., Munday et al., 2011; Bignami et al., 2013; Frommel et al., 2013; Perry et al., 2015). A recent meta-analysis lends some support to this theory by showing that mortality increased in pelagic species under elevated CO₂, whereas mortality often decreased in benthic species, which experience higher and more variable CO₂ compared with pelagic species (Cattano et al., 2018). Our results support this as *A. polyacanthus* is a benthic reef species that shelters within the reef-matrix at night where it is likely exposed to CO₂ levels considerably higher than in the surrounding water column (e.g., Albright et al., 2013; Kline et al., 2015).

While natural CO₂ cycles have been used to explain tolerance to future OA conditions in marine fish, very few studies have incorporated them in their experimental designs (but see Ou et al., 2015; Jarrold and Munday, 2018). Here, we show that diel CO₂ cycles had a significant positive effect on growth of juvenile *A. polyacanthus* under elevated CO₂. These beneficial effects were most apparent for weight wet, with fish reared under both the ±300 and ±500 μatm diel-cycling CO₂ regimes being significantly heavier compared to fish reared under stable elevated CO₂. We observed a similar positive effect of diel CO₂ cycles on standard length, although there was only a significant difference between the ±300 μatm diel-cycling and the stable elevated CO₂ treatments. These results suggest that the positive effects of diel CO₂ cycles are magnitude dependent, with greater effects observed under the smaller ± 300 μatm diel-cycling CO₂ regime compared with ±500 μatm CO₂. This may reflect the closer match of the ±300 μatm CO₂ treatment to the diel fluctuations these fish experience on the reef. Similar trends to those described above were observed in a previous study (Jarrold and Munday, 2018) carried out the same species and in which the same pCO₂ treatments were used, although no significant differences were detected in that study, most likely due to the lower levels of replication (three clutches compared to the four in this study) (see **Supplementary Table 1** for a summary comparison between the current and past studies). Positive effects of diel CO₂ cycles on growth under elevated CO₂ were also reported in the freshwater stage of larval pink salmon, *O. gorbuscha*, where the negative effect that 1000 μatm CO₂ had on length was absent in the diel-cycling treatment (450–2000 μatm) (Ou et al., 2015).

The underlying physiological mechanism responsible for our observed positive effect of diel CO₂ cycles on growth under elevated CO₂ is unknown. However, our results and those of Ou et al. (2015) suggest that it is energetically less expensive for fish to live in a diel-cycling elevated CO₂ environment compared to a stable elevated CO₂ environment. Aerobic scope [the difference between resting metabolic rates (RMR) and maximal metabolic rates (MMR)] represents the energy budget available for an organism to undertake aerobic tasks (e.g., growth). Previous work has shown that exposure to stable elevated CO₂ can alter metabolic rates in some fish, highlighting an increased cost of living (Munday et al., 2009a; Enzor et al., 2013; Strobel et al.,

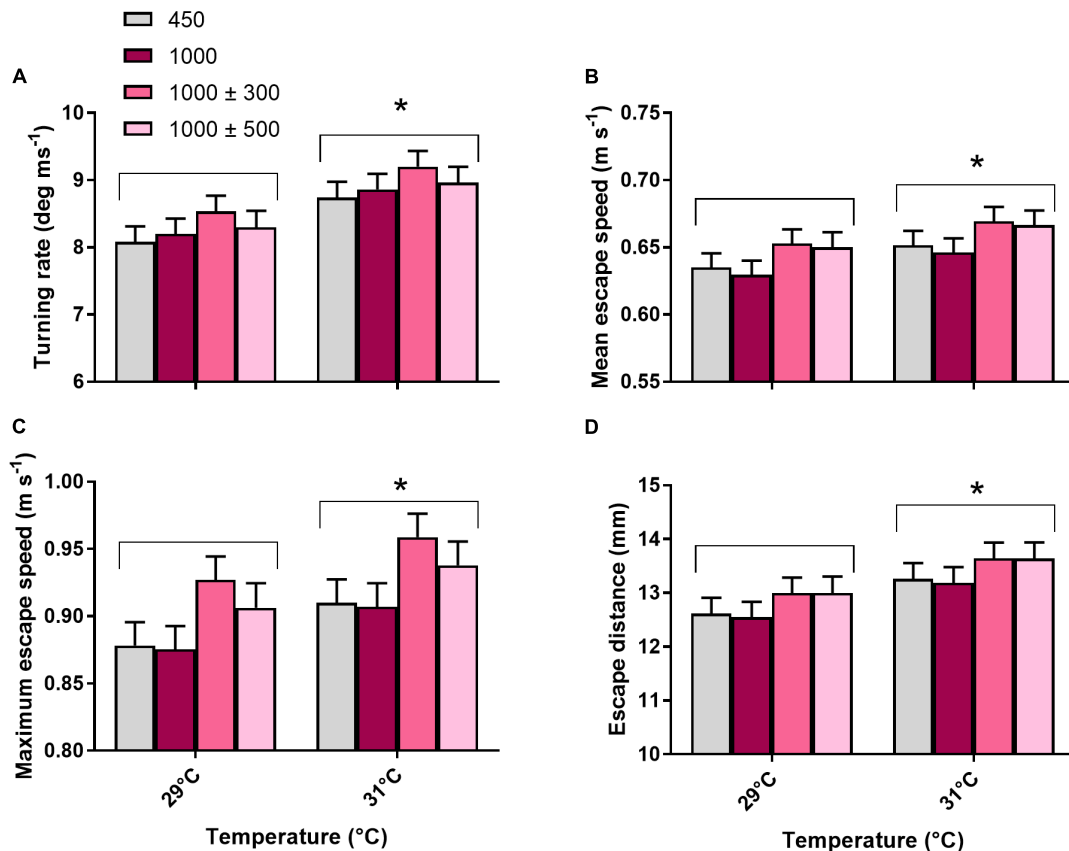


FIGURE 6 | Effects of elevated CO₂ and temperature on locomotor fast start traits of juvenile *A. polyacanthus*. **(A)** Turning rate. **(B)** Mean escape speed. **(C)** Maximum escape speed. **(D)** Escape distance. Bars represent means ± SE. *Indicates a significant difference between temperature treatments ($P < 0.05$).

2013; Ou et al., 2015; Cattano et al., 2018; Laubenstein et al., 2018). Thus, we suggest that the positive effects of diel CO₂ cycles on growth could be driven by increased aerobic scope under such conditions. In line with this, Ou et al. (2015) reported that maximal metabolic rates (MMR) of larval pink salmon after transition to saltwater were negatively impacted by stable elevated CO₂ (1600 μatm), but not by diel-cycling conditions (450–1600 μatm), with resting metabolic rates (RMR) being similar in both treatments. However, because the mean CO₂ also differed between the stable and fluctuating treatments in that experiment it is not possible to be certain whether the changes in MMR were being driven primarily by the cycling CO₂ regime or change in mean CO₂. Alterations in aerobic capacity under elevated CO₂ may be linked to the increased metabolic costs associated with defending acid-base status (Perry and Gilmour, 2006; Baker and Brauner, 2012). Thus, we hypothesize that the cost of acid-base regulation under elevated CO₂ is reduced by the presence of diel CO₂ cycles, ultimately resulting in more energy available for life-history processes such as growth.

Temperature Effects

Elevated temperature (31°C) negatively impacted both survival and growth of juvenile *A. polyacanthus*. This demonstrates that 31°C is above the optimal temperature for this species

and is consistent with previous findings (Munday et al., 2008; Rodgers et al., 2018). The negative impacts of elevated temperature on growth and survival were most likely correlated to increased energetic demands associated with increased metabolic costs. Indeed, previous work on coral reef fish, including *A. polyacanthus*, has shown that exposure to 31°C causes a significant increase in RMR and subsequent decrease in aerobic scope (Munday et al., 2009a; Nilsson et al., 2009; Donelson et al., 2012). While elevated temperature (31°C) had an overall negative effect on survival of juvenile *A. polyacanthus*, CO₂ treatment dependent effects were present. There was a trend of decreased survival in all the CO₂ treatments at 31°C; however, significant differences were only observed in the two diel-cycling elevated CO₂ treatments. Most mortality was observed in the mornings, indicating that deaths were occurring at night during the high CO₂ peaks, within the first week of the experiment. These observations suggest that for newly hatched fish, which might not have fully developed acid-base regulation capacity, and for which the cost of homeostasis is greater (Brauner, 2009), the metabolic demands to tolerate the higher CO₂ levels caused by diel cycles become too great under elevated temperature. The effects of elevated temperature on fish early life growth have been varied. For example, temperatures above the natural preference range were shown to enhance

growth of larval yellowtail kingfish, *Seriola lalandi* (Watson et al., 2018). In contrast, elevated temperature negatively impacted the growth and survival of larval Atlantic herring, *Clupea harengus*, despite the elevated temperature still being within the species preferred thermal range (Sswat et al., 2018). Differences between studies could potentially be linked to food availability, with studies reporting positive effects usually providing a continuous supply of high-density food that enabled energetic demands to be met. In this study, juvenile *A. polyacanthus* were fed a fixed ration of food daily, however, previous work on this species has shown that the effects of 31°C on growth were no different depending on whether fish were fed a high or low quality diet (Munday et al., 2008). Thus, we are confident that our feeding regime had little impact on the observed response.

Behavior

CO₂ Effects

Behavioral abnormalities in fish under OA conditions are thought to be linked to the effects of acid-base regulation on the function of type A γ -aminobutyric acid (GABA_A) neurotransmitter receptors which have specific conductance for HCO₃[−] and Cl[−] (Nilsson et al., 2012; Hamilton et al., 2014; Heuer et al., 2016). Under elevated CO₂ fishes increase intracellular and extracellular HCO₃[−] concentrations, with a corresponding decrease in Cl[−], to prevent plasma and tissue acidosis (Wood et al., 1990; Baker et al., 2009; Heuer and Grosell, 2014). This altered ion gradient is thought to turn some GABA_A receptors from inhibitory to excitatory, ultimately leading to behavioral impairments (Heuer et al., 2016). Importantly, acid-base regulation in fish is under circadian control (Peterson and Gilmore, 1988). In a recent study on *A. polyacanthus*, variation in behavioral tolerance to stable elevated CO₂ was linked to the differential expression of genes related to circadian rhythm control and indicated that behaviorally sensitive fish may have been displaying more pronounced acid-base regulation (Schunter et al., 2016). This suggests that diel CO₂ cycles, and associated circadian rhythms, are likely to have an important influence on the sensitivity of reef fishes to elevated CO₂.

Consistent with previous work, we observed that absolute lateralization of juvenile *A. polyacanthus* was significantly reduced under stable elevated CO₂ (Welch et al., 2014; Jarrold et al., 2017). Similar results have been reported in a range of other species (Domenici et al., 2012; Jutfelt et al., 2013; Green and Jutfelt, 2014; Lopes et al., 2016; Schmidt et al., 2017). Furthermore, in agreement with a past study, we show that the severity of behavioral impairment under elevated CO₂ is reduced by the presence of diel CO₂ cycles, with partial restoration in the smaller diel-cycling treatment and full restoration in the larger diel-cycling treatment (Jarrold et al., 2017; see **Supplementary Table 1** for a summary comparison between studies). This is an important finding given the ecological consequences of behavioral abnormalities and that past research has shown a limited capacity for acclimation/adaptation of behavioral traits to stable elevated CO₂ (Welch et al., 2014; Welch and Munday, 2017). Our lateralization results suggest that GABA_A receptors were functioning more normally under elevated CO₂ when a diel

cycling CO₂ regime was present. This could indicate that fish kept under diel CO₂ cycles underwent less acid-base compensation compared to those reared under stable elevated CO₂. For coral reef fish it appears that complete acid-base regulation in the brain under stable elevated CO₂ levels takes between 24 and 96 h, as this is the exposure period required before behavioral abnormalities manifest (Munday et al., 2010). Our results suggest that for fish reared under diel-cycling CO₂, exposure to lower CO₂ levels for several hours each day is enough to prevent the physiological changes that would normally occur at a stable high CO₂ (see discussion in Jarrold et al., 2017 for more detail). It is possible that changes in acid-base regulation under diel-cycling elevated CO₂ is linked to altered expression of circadian rhythm genes. Further work is needed to determine if and how acid-base regulation is altered under elevated CO₂ when diel CO₂ cycles are present.

Routine activity and boldness of juvenile *A. polyacanthus* were unaffected by exposure to stable elevated CO₂, which is consistent with some previous work (Nowicki et al., 2012; Heinrich et al., 2016; Sundin et al., 2017). Yet, these observations contrast with other studies on coral reef fish where significant effects of elevated CO₂ on routine activity have been documented (Munday et al., 2010, 2014; Cripps et al., 2011; Devine et al., 2012; McCormick et al., 2013). Instead, our findings are more consistent with what has been observed in non-tropical species (Maneja et al., 2015; Sundin and Jutfelt, 2015; Duteil et al., 2016; Schmidt et al., 2017). The underlying explanation for discrepancies between studies is unclear but may be related to methodological differences. In general, the studies on coral reef fish which have reported significant effects of stable elevated CO₂ on activity/boldness tested fish in the field on natural reef structures, or in an artificial tank environment which had a shelter. In contrast, many of the studies reporting no effects, including this one, conducted open field tests in an artificial environment, generally, without any shelter and/or with minimal acclimation time. Thus, it could be that without the option of a 'safe place' and/or insufficient acclimation time the impacts of elevated CO₂ are masked by the effects of a general stress response.

While routine activity was unaffected by stable elevated CO₂, it was altered by the presence of a ± 500 μ atm CO₂ diel cycle, with fish in the cycling elevated CO₂ treatment being less active (−10.2%) and swimming slower (−15.9%) compared to controls. Impaired metabolic performance can likely be ruled out as a potential underlying mechanism for the observed decrease in routine activity because increased growth was observed under these conditions. We propose that this decrease in routine activity was associated with altered anxiety levels. Activity in a novel environment can be an indicator of anxiety, whereby reduced activity is usually associated with increased anxiety (Egan et al., 2009; Maximino et al., 2010). Increased anxiety/reduced boldness in stable elevated CO₂ conditions has been observed in juvenile Californian rockfish, *Sebastes diploproa*, (Hamilton et al., 2014) and three-spined stickleback, *Gasterosteus aculeatus*, (Jutfelt et al., 2013), but why we only observed altered anxiety in the largest diel-cycling elevated CO₂ treatment is unclear. Anxiety in fish is linked to GABA_A receptor functioning, with normal function acting to reduce anxiety (Stewart et al., 2011; Hamilton et al., 2014). Our behavioral lateralization data and previous work

(Jarrold et al., 2017), where diel CO₂ cycles had a positive effect on behavior, suggests that GABA_A receptor functioning is closer to normal under elevated CO₂ when diel CO₂ cycles are present. Thus, it appears that another physiological mechanism, other than the GABA_A pathway, may be responsible for our observed reduced activity in the 1000 ± 500 µatm CO₂ treatment. One possible explanation is that the higher CO₂ peaks may have altered stress hormone levels, such as cortisol, which are known to directly correlate with anxiety behaviors in fish (Egan et al., 2009; Cachat et al., 2010). The link between behavioral responses and stress hormones in fish under elevated CO₂ conditions is currently unknown and would be an interesting area for future work.

All fast start non-locomotor and locomotor variables in juvenile *A. polyacanthus* were unaffected by exposure to elevated CO₂, regardless of whether a stable or cycling regime was experienced. This contrasts with most previous studies on coral reef fish where significant effects of elevated CO₂ on fast starts have been observed (Allan et al., 2013, 2014; Munday et al., 2016). Negative effects of elevated CO₂ on fast start performance were also observed in the temperate species yellowtail kingfish, *S. lalandi* (Watson et al., 2018). Our results are comparable to those reported for the coral reef damselfish *Pomacentrus wardi*, where elevated CO₂ was shown to have no effect on escape speed and distance (Allan et al., 2017). The methods and CO₂ levels used in all studies, including this one, were very similar and so differences are most likely due to species-specific responses.

Temperature Effects

Elevated temperature had a clear negative effect on routine activity and boldness of juvenile *A. polyacanthus*. These results contrast with previous experiments on coral reef damselfish where activity and boldness were generally shown to increase at higher temperatures (Biro et al., 2010; McCormick and Meekan, 2010). However, the temperatures used in these studies were within the species preferred thermal range. When this is the case fish become more active to seek food so that increased metabolic costs associated with higher temperatures can be met (Biro et al., 2010). In contrast, when temperatures exceed a species optimum, as was the case in this study, reduced activity could represent an energy-saving strategy (Johansen and Jones, 2011). Additionally, elevated temperatures have been shown to increase stress hormone levels in fish (Davis, 2004) which is potentially another mechanism driving the observed reduction in activity and boldness, although additional testing is needed to confirm this.

In contrast to routine activity, elevated temperature had a small positive effect (4–8%) on fast start locomotor variables. Allan et al. (2017) also showed that elevated temperature had a stronger effect on fast start performance compared to elevated CO₂, although in this case the effects were negative. Watson et al. (2018) observed enhanced fast start performance in larval yellowtail kingfish at temperatures above their preferred range, although this was attributed to increased larval size under elevated temperature. While elevated temperature had a negative effect on size of juvenile *A. polyacanthus* in this study there was no difference in the size of fish selected for

fast start trials between treatments. The increased locomotory performance observed in this study may therefore have been linked to the combined effects of reduced viscosity and increased muscle efficiency in warmer water (Fuiman and Batty, 1997; Wieser and Kaufmann, 1998). The underlying reason for the opposing effects elevated temperature had on routine activity and fast starts is unknown but may be related to the fact routine activity is under aerobic control whereas fast starts are driven by anaerobic metabolism (Domenici and Blake, 1997).

Summary

Diel CO₂ cycles had a significant effect on three ecologically important traits in *A. polyacanthus*. Firstly, diel CO₂ cycles were shown to mediate the negative effects of elevated CO₂ on absolute lateralization, confirming results of a previous study. Secondly, diel CO₂ cycles had a significant positive effect on growth under elevated CO₂. Finally, routine activity of fish from the larger diel-cycling CO₂ treatment was significantly lower compared to control fish. Importantly, these effects were observed at both current-day and future elevated temperatures, demonstrating that higher temperature did not significantly alter the effects of diel CO₂ cycles in the traits measured here. These findings highlight the importance of incorporating natural CO₂ variability in experiments to more accurately assess the impacts of future OA on shallow water coastal organisms (Cornwall et al., 2013; Frieder et al., 2014; Ou et al., 2015; Jarrold et al., 2017; Wahl et al., 2018).

In contrast to elevated CO₂, elevated temperature had a significant effect on every trait except behavioral lateralization. Furthermore, in all cases when a trait was significantly affected by both climate change drivers' temperature had the strongest affect. While this is unsurprising for survival and growth, it is perhaps unexpected for behavioral traits where elevated CO₂ has often been shown to have a significant impact. Our results suggest that behavioral responses that involve decision making (e.g., lateralization and response to cues) may indeed be more impacted by elevated CO₂, whereas responses which are governed by energetic constraints (e.g., activity, fast starts and competitive interactions) will be more impacted by elevated temperature. This is consistent with other recent studies where elevated temperature had a stronger effect on fast start performance and swimming activity compared to elevated CO₂ (Allan et al., 2017; Watson et al., 2018).

A limitation of this study was that we were unable to include a diel-cycling control treatment. It is possible that fish reared under a diel-cycling control may perform differently than to those reared under a stable control (Cornwall et al., 2013). For example, as was observed under elevated CO₂, it is possible that fish reared under a diel-cycling control may perform better compared to those reared under a stable control and thus the negative effects of elevated CO₂ may still be present. However, based on our current understanding of the impacts elevated CO₂ has on fishes we do not believe this would be case, although further work is needed to confirm this. In this study we only incorporated diel cycles with elevated CO₂ as this was a main stressor of

interest. However, shallow water habitats also experience diel cycles in temperature (e.g., Kline et al., 2015), which are also known to affect fish performance (e.g., Hokanson et al., 1977). Importantly, the highest level of temperature and CO₂ cycles occurs at opposite times of the day, with CO₂ cycles having their high peak during the night and temperature cycles having their high peak during the day. Thus, the responses of fish to a cycling CO₂ and temperature environment could be different to a static one, or when one stressor is static as was the case in this study. Future experiments should incorporate both diel-cycling CO₂ and temperature to further our understating on how marine fish will respond to climate change.

AUTHOR CONTRIBUTIONS

MJ and PM contributed to conception and design of the study. MJ performed the experiments and analyses. MJ wrote the first draft of the manuscript with input from PM.

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FUNDING

This project was funded by the Australian Research Council (PLM). MJ was supported by an International Postgraduate Research Scholarship.

ACKNOWLEDGMENTS

We thank the SeaSim team for their technical support throughout the experiment at AIMS. Additionally, we thank Rhondra Jones for statistical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00458/full#supplementary-material>

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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